

Simian Virus 40 DNA Sequences in DNA of Healthy Adult Mice Derived from Preimplantation Blastocysts Injected with Viral DNA

(blastocyst microinjection *in vitro*/development/DNA reassociation kinetics of simian virus 40)

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ABSTRACT Explanted mouse blastocysts were microinjected in the blastocoel cavity with simian virus 40 (SV40) viral DNA. After surgical transfer to the uteri of pseudopregnant surrogate mothers, approximately 40% of the blastocysts developed to term and became healthy adults without apparent tumors at 1 year of age. Molecular hybridization tests for the presence of SV40-specific DNA sequences were conducted on DNA extracted from various organs of these animals. Between 0.5 and 13 SV40 genome equivalents per diploid mouse DNA value were found in some organs of approximately 40% of the adult survivors; this represents a substantial augmentation of the amount administered per embryo. The results are consistent with the working hypothesis that the SV40 DNA may have been integrated into the host genome; alternatively, the viral DNA may have replicated as an extrachromosomal entity or by lytic infection in a few permissive cells. Persistence of the viral DNA from preimplantation stages to adult life may thus provide a new tool for experimental investigation of vertical transmission and expression of tumor viruses.

Evidence for possible genetic transmission of some RNA tumor viruses from parent to offspring has been presented (1) and tumor virus DNA sequences have been found in normal and tumor tissues and in transformed and untransformed tissue culture cells (2). Such observations have led to the hypothesis that vertebrates may commonly contain and vertically transmit RNA tumor virus genomes (3). Nucleic acid hybridization studies have also revealed viral-specific sequences in tissue culture cells transformed by DNA tumor viruses (4-6), but no indication of genetic transmission of DNA-containing viruses has yet been obtained.

In order to investigate the mechanism of vertical transmission of tumor viruses and their possible activation during later life, a useful approach would be to infect the zygote or early embryo with virus under controlled conditions and to follow the consequences of this infection in the resultant individual. The mouse is an especially favorable species for such an approach, as the preimplantation stages can be manipulated and cultivated *in vitro* and then transferred to a foster recipient for further development to term. In previous explorations with viral agents along these lines, mouse eggs have been experimentally infected with adenovirus (7) or Mengo virus (8), but rapid death of the embryo resulted. On the other hand, infection with Moloney sarcoma virus

or simian virus 40 (SV40) seemed not to block *in vitro* development of mouse embryos from the two-cell to the blastocyst stage and both these viruses could be rescued from the infected embryos at that time (9). When SV40 DNA rather than the virus itself was used, development of two-cell embryos but not of morulae to the blastocyst stage was inhibited. No postimplantation SV40-containing survivors have been reported prior to the present study.

In the preceding work, the embryos were incubated in solutions containing virus or viral DNA. We have, instead, administered purified SV40 viral DNA by microinjection into preimplantation mouse blastocysts. We report here the successful infection and development of such embryos into mature and apparently healthy animals, and the experimental recovery of SV40-specific DNA sequences from tissues of the adults.

MATERIALS AND METHODS

SV40 DNA. Viral DNA was isolated and purified by sedimentation through alkaline sucrose gradients as described previously (10). The purified DNA was precipitated with ethanol and resuspended at a concentration of 1000 $\mu\text{g}/\text{ml}$ in phosphate-buffered saline (0.15 M NaCl, 5 mM dextrose, 1 mM sodium citrate, 20 mM sodium phosphate, pH 7.4).

Isolation of Embryos. Three-week-old females of the C3H inbred strain were superovulated with hormone injections as reviewed elsewhere (11), mated to adult males of the same strain, and checked for vaginal plugs the next day. Embryos were flushed from the oviducts a day later at 2- to 4-cell stages and cultured in Brinster's medium (12) for 2 days until they reached the blastocyst stage (approximately 32-64 cells).

Microinjection. Blastocysts were microinjected essentially as described by Lin (13). This consisted of first immobilizing the embryo, by means of gentle suction, on the smoothly polished blunt tip of a small-bore "egg-holding" micropipette held in a micromanipulator (Leitz); the material to be injected was then introduced into the embryo in a sharp-pointed micropipette held in another unit of the apparatus. Purified SV40 DNA (20 μl) was placed on a plastic petri dish and the embryos to be injected were added in approximately 20 μl of Brinster's medium and immediately covered with paraffin oil (previously equilibrated with medium). Some of the SV40 DNA-containing medium around the embryo was then taken up in a micropipette and injected into the blastocoel cavity of

Abbreviations: SV40, simian virus 40; EDTA, ethylenediamine-tetraacetic acid.

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the blastocyst. The SV40 concentration of the injected suspension was approximately 500 $\mu\text{g}/\text{ml}$. This allowed an injection of a rather high concentration: 2–10 pg or 4 to 20 $\times 10^5$ DNA molecules per embryo.

The injected embryos were then promptly placed in culture medium and were subsequently transferred surgically to the uterine horns of a pseudopregnant recipient mated to a vasectomized male a day later than the matings of the egg donors (11).

Extraction of Mouse DNA. The mice were starved for 2 days before being sacrificed and the liver, kidneys, and brain were removed. The tissues were minced and homogenized, using a motor-driven Dounce homogenizer. The cells were lysed in 0.01 M NaCl, 0.01 M Tris·HCl, pH 7.4, 0.01 M ethylenediaminetetraacetic acid (EDTA), 4% sodium dodecyl sulfate; 1 M NaClO₄ was added subsequently. The DNA was extracted four times, using the chloroform-isoamyl alcohol procedure (14). Three volumes of ethanol were added and the DNA was spooled around a glass rod, resuspended in 0.01 M NaCl, 0.01 M Tris·HCl, pH 7.4, 0.01 M EDTA, and digested with RNase (20 $\mu\text{g}/\text{ml}$) for 45 min at 37°. After the salt concentration had been adjusted to 0.15 M NaCl, the DNA was extracted with CHCl₃ for an additional four times, spooled on a glass rod, and finally resuspended in 0.01 Tris, HCl, pH 7.4, 1 mM EDTA.

Some DNA preparations were subsequently banded in ethidium bromide–CsCl gradients (1.565 g of CsCl/ml, 1 mM EDTA, 10 mM Tris·HCl, pH 7.4, 100 $\mu\text{g}/\text{ml}$ of ethidium bromide; centrifugation was at 52,000 rpm for 16 hr in the type 60 Ti fixed-angle Spinco rotor).

The A_{260}/A_{280} ratio of a typical DNA preparation was between 1.9 and 1.95.

Calf-thymus DNA was purchased from Sigma and purified as described above.

Preparation of Highly Labeled SV40 DNA. Unlabeled SV40 DNA was isolated by sedimentation through alkaline sucrose gradients (10), banded in ethidium bromide–CsCl, and finally sedimented through a neutral sucrose gradient. This DNA was used as a template for *Escherichia coli* polymerase I to synthesize ³²P-labeled SV40 DNA *in vitro*. As a radioactive precursor, ³²P-labeled dCTP was synthesized (15) at a specific radioactivity of 1000 Ci/mmol. The reaction mixture contained all four dNTP's, SV40 DNA, pancreatic DNase, and *E. coli* DNA polymerase I (16) which was a gift of Dr. Arthur Kornberg. This *in vitro* nick translation reaction will be described in detail (17).

The DNA synthesized had a specific radioactivity of 1 to 6 $\times 10^8$ cpm/ μg and sedimented with 5.5 S in alkaline sucrose gradients. The size of the *in vitro*-synthesized DNA was monitored at the beginning and at the end of each hybridization experiment and was found to remain essentially unchanged. DNA of this high specific activity was not used later than 8 days after synthesis.

The radioactive SV40 DNA synthesized *in vitro* was double-stranded. When heat-denatured and reannealed in the presence of cold SV40 DNA, it reannealed to 95–100% as shown by resistance to nuclease SI. The reannealing kinetics had the same $C_{0t}/2$ (18) as *in vivo*-synthesized and labeled SV40 DNA.

Molecular Hybridization. ³²P-Labeled SV40 DNA (3 to 10 $\times 10^{-4}$ $\mu\text{g}/\text{ml}$) was added to a reaction mixture containing

300–1000 $\mu\text{g}/\text{ml}$ of mouse- or calf-thymus DNA in 0.01 M Tris·HCl, pH 7.4, 1 mM EDTA. The DNA mixture was sonicated for 30 sec in a Branson sonifier at a setting of 1, using the microtip, boiled for 10 min, and chilled in ice. NaCl was added to a final concentration of 1 M, and the solution was overlaid with paraffin oil and incubated at 68°. Samples of 30–100 μl were removed at different times with a Hamilton syringe and diluted into 1 ml of cold SI digestion buffer (0.1 M Na acetate, pH 4.5, 0.01 M NaCl, 600 μM ZnCl₂) containing 40 $\mu\text{g}/\text{ml}$ denatured and 5 $\mu\text{g}/\text{ml}$ native calf-thymus DNA. The total radioactivity removed at one time point was between 1000 and 3000 cpm. The samples were digested with SI nuclease (19) for 30 min at 37°, precipitated with 10% trichloroacetic acid, and counted for SI-resistant radioactivity.

Plotting of Data. The reciprocal of the fraction of labeled SV40 DNA remaining single-stranded ($[c_0/c_0 - c_a]$; c_0 = fraction of single-stranded DNA at time 0, c_a = fraction of DNA annealed at indicated time) is plotted as a function of time of hybridization. This plot results in a straight line, the slope of which is directly proportional to the concentration of the DNA reannealing (20). The number of SV40 genome equivalents in the DNA extracted from mouse organs was calculated according to methods previously described (5).

RESULTS

Development after SV40 Injection. In a total of three series, 80 embryos were injected with SV40 DNA and transferred to six surrogate mothers. Thirty-one mice were born, one of which died shortly after birth. Another animal died suddenly at the age of 9 weeks and could not be autopsied before autolysis was advanced. The remaining 29 mice continued to appear healthy; when they were killed at 1 year of age, no tumors or other abnormalities were found. The survival rate to birth was therefore 39% and the long-term survival rate was 36%. These results are comparable to survival of uninfected embryos after *in vitro* manipulations and transfer to foster mothers (11, 13).

SV40-Specific Sequences in the Extracted Mouse DNA. DNA was extracted from some of the tissues of 25 of the 29 long-range experimental survivors. Liver and kidneys from each of these were pooled and tested; in addition, the brain was tested from 15 of the 25. The DNA of pooled liver and kidneys was also isolated from each of 60 control mice randomly taken from the animal colony; these included 35 animals of the same inbred strain as the experimentals (C3H) and 25 of the ICR randombred strain.

Detection and measurement of SV40 sequences in these DNA preparations was accomplished by comparing the reassociation kinetics of highly labeled denatured SV40 DNA in the presence of DNA extracted from experimental animals, control animals, or calf thymus. An increase in the rate of reassociation of the radioactive probe DNA indicates the presence of viral-specific sequences in the DNA under investigation.

The results obtained with DNA extracted from liver and kidneys or from brains of infected animals are shown in Figs. 1 and 2. The reciprocal of the fraction of labeled SV40 DNA remaining single-stranded is plotted as a function of time of hybridization. The rate of reannealing was accelerated by some mouse DNA preparations of putatively infected ani-

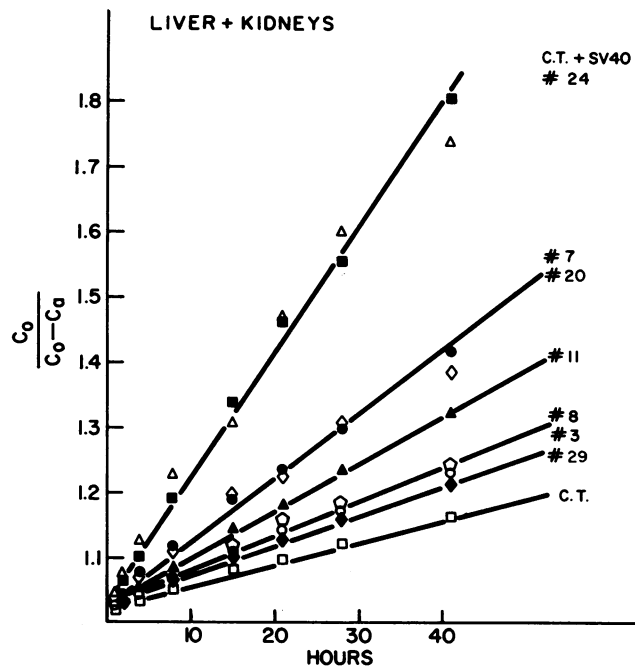


FIG. 1. Reassociation kinetics of ^{32}P -labeled SV40 DNA in the presence of unlabeled DNA extracted from pooled liver and kidneys of each of 7 (numbered) mice derived from blastocysts injected with SV40 DNA. The reaction mixtures, containing 0.5 ng/ml of ^{32}P -labeled SV40 DNA (1.6×10^8 cpm/ μg), 680 μg /ml of mouse- or calf-thymus DNA in 0.01 M Tris-HCl, pH 7.4, 1 mM EDTA, 1 M NaCl, were heat-denatured and incubated at 68° . Samples were removed at different times and the fraction of SV40 DNA reannealed was determined by digestion with SI nuclease. The results are plotted as the reciprocal of DNA remaining single-stranded as a function of time. Calf-thymus DNA (= C.T.) and calf-thymus DNA plus 1.8 ng/ml of unlabeled SV40 DNA (= C.T. + SV40) were included as controls. Each point represents a total of 2000 cpm.

mals or when unlabeled SV40 DNA was added to calf-thymus DNA, as compared to DNA of control mice or calf-thymus DNA alone. Seven of the 25 liver-kidney and 4 of the 15 brain DNA preparations tested were positive for SV40 DNA sequences; the 10 animals in which one or both classes of tissue samples were positive are listed in Table 1. These results demonstrate that SV40-specific information can be traced in slightly over one-fourth of the organs tested from mice derived from SV40 DNA-injected blastocysts. The tissue DNA of the control mice behaved indistinguishably from calf thymus DNA and was found not to increase the reannealing kinetics of denatured SV40 DNA (Fig. 3).

The numbers of viral copies per mouse genome equivalent were calculated as described elsewhere (5) and are summarized in Table 1. The number of SV40 copies is expressed with reference to the diploid mouse DNA value rather than on a per cell basis, because of the presence of unknown numbers of polyploid and binucleate cells in some of the tissues tested. Pooled liver and kidney DNA preparations contained between 0.5 and 3.6 SV40 equivalents per diploid mouse DNA value and between 0.7 and 13 copies were found in the brain DNA preparations. In most cases, where both brain and pooled liver and kidney DNA preparations were tested separately, only one tissue (or tissue pool) contained SV40 DNA sequences, while the other did not.

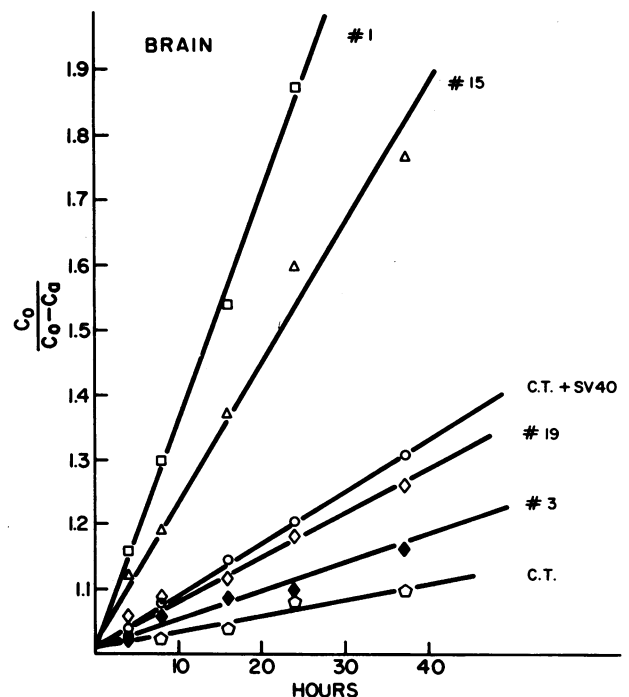


FIG. 2. Reassociation kinetics of ^{32}P -labeled SV40 DNA in the presence of unlabeled DNA extracted from the brains of four (numbered) mice derived from blastocysts injected with SV40 DNA. The reaction mixtures containing 0.3 ng/ml of ^{32}P -labeled SV40 DNA (1.4×10^8 cpm/ μg) and 310 μg /ml of unlabeled mouse- or calf-thymus DNA were reannealed as described in Fig. 1. Calf-thymus DNA plus 0.7 ng/ml of unlabeled SV40 DNA were included as controls. Each point represents a total of 1000 cpm.

DISCUSSION

At the time of injection, the blastocyst consists of some 32 to 64 or more cells of which roughly three-fourths form an enveloping layer—the trophoblast—at the surface of the sphere; the rest comprise the internally situated small group

TABLE 1. Detection of SV40 DNA in DNA extracted from various mouse organs

Mouse no.	No. of SV40 copies per diploid mouse DNA value	
	Liver and kidneys	Brain
1	0	13
3	0.62	0.7
7	1.5	0
8	0.72	n.t.
11	1.0	n.t.
15	0	8.5
19	0	1.95
20	1.6	0
24	3.6	n.t.
29	0.5	0

The number of SV40 genome equivalents in the DNA extracted from these mouse organs was calculated according to previous methods (5) from the data in Figs. 1 and 2, for each of the 10 mice with evidence of SV40 after injection of SV40 DNA in the preimplantation blastocyst stage. Liver and kidneys were pooled from each of 25 mice tested; brains of 15 of the 25 were tested. n.t. = not tested.

known as the inner cell mass. The latter is located to one side of a fluid-filled cavity, the blastocoel, into which the SV40 DNA was injected. The embryo proper will ultimately develop from only some [possibly very few (21)] cells of the inner cell mass; the remainder, and the trophoblast cells, will form extraembryonic and placental tissues. Thus, administration of viral DNA by microinjection rather than by the previous method (9) of incubation in DNA, has the advantage of allowing a high concentration (see *Materials and Methods*) of DNA in direct contact with at least some of the inner cell mass cells, without disturbing the physiological normalcy of the outside incubation milieu. Such an arrangement might be expected to enhance uptake and possible chromosomal integration in the future embryo cells of the inner cell mass. At the same time, one would expect that some, but not necessarily all, of the prospective embryo cells in the inner cell mass might become infected.

The results demonstrate that SV40 DNA sequences can in fact survive through at least a substantial part of the animals' lifespan in the cells of mice injected in the blastocoel at the blastocyst stage. The occurrence of detectable virus-specific DNA in some organs and not others of a given animal (Table 1) is compatible with the possibility that some, rather than all, of the cells of the embryo proper in the inner cell mass had become infected. It is also possible that infection was established at a point in development beyond the blastocyst stage. If, as generally believed, mouse cells are not a permissive host for SV40 (22), virus particles could not have been synthesized and it seems improbable that the naked viral DNA could have survived for any appreciable period of time without being integrated into the host genome or replicated. However, it is not ruled out that the cells, or a small percentage of them, might be permissive at this early stage of development; if so, infection could have been delayed and some cells would be unaffected. Alternatively, all the embryo cells may have been infected initially with viral DNA, but the virus information could have been eliminated from the majority of them during subsequent development.

Quantitation of SV40 copies per diploid DNA value showed a wide variation: between 0.5 and 3.6 copies per diploid DNA value were found in the DNA preparations from pooled liver and kidneys, and between 0.7 and 13 copies in the brains. The considerably greater apparent variation in the number of SV40 copies, and the higher number of copies, found in the brain as compared with liver and kidney DNA may be due at least in part to actual absence of SV40 sequences in one of the two pooled organs (liver or kidney), thus lowering the number of SV40 copies calculated in the pooled preparations and resulting in a smaller variation than in organs (i.e., brain) tested individually. Another possibility is that SV40 DNA may have a better chance of being conserved in brain as opposed to liver or kidney. Further experiments with a greater variety of individually tested tissues of mice from infected blastocysts would clarify these questions. In any event, the SV40 DNA actually detected in the adult tissues represents an augmentation of the input by at least two orders of magnitude (on the basis of 1 copy of SV40 DNA/diploid mouse genome being equivalent to approximately 1 ng SV40/mg of cell DNA, and a liver-plus-kidney preparation yielding 2–3 mg of DNA and a brain 100–200 μ g).

In SV40-transformed cultured cells, viral-specific sequences have been found to be covalently linked to host sequences

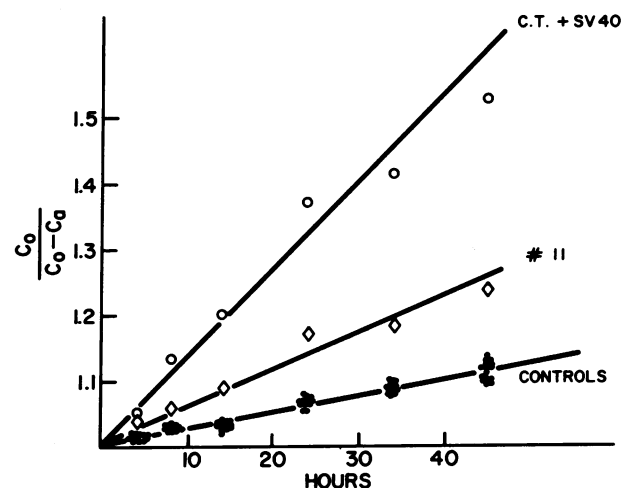


FIG. 3. Reassociation kinetics of 32 P-labeled SV40 DNA in the presence of unlabeled DNA extracted from control mice. The reaction mixtures contained 0.4 ng/ml of 32 P-labeled SV40 DNA (1.1×10^8 cpm/ μ g) and 800 μ g/ml of mouse- or calf-thymus DNA. Reannealing was performed as described in Fig. 1. The number refers to a single experimental mouse from an SV40 DNA-injected blastocyst; C.T. + SV40 denotes calf-thymus DNA plus 1.7 ng/ml of unlabeled SV40 DNA. Each point represents a total of 1800 cpm.

(4). Therefore, the most appealing interpretation to explain the presence of SV40-specific sequences in adult mice following infection at a preimplantation stage would be to assume that the viral DNA was integrated at this early stage of development into the host genome and was thus conserved during further development. However, our results do not exclude an extra-chromosomal existence of the SV40 genome, for example, as an independently replicating plasmid, or as a lytic infection in a few permissive cells. We therefore plan to investigate whether the SV40-specific information can be genetically transmitted from the infected mice to their offspring; integration would be proven if transmission of SV40 DNA occurred in accordance with simple Mendelian expectations.

The injection of mouse blastocysts with purified SV40 DNA did not ostensibly interfere with normal development of the embryos to healthy adult mice which were still tumor-free at 1 year of age. This was not due to the trivial possibility that the viral DNA did not successfully infect and was eliminated from the injected embryos, as virus-specific DNA sequences were detected in 10 of 25 infected year-old animals, or in about 25% of DNA preparations extracted from some of their tissues (Table 1). It is nevertheless possible that the animals may not have been old enough to exhibit tumorigenesis of SV40 origin; to test this possibility, the experiment will have to be repeated for longer survival periods.

The absence of any obvious signs of expression of viral genetic functions, i.e., tumor formation, up to one year of age of the host is reminiscent of the "cryptic transformants" described earlier (23) which harbor SV40 information but behave essentially like normal untransformed cells. Whether partial virus gene expression can occur in infected mice on the transcriptional or translational level is presently an open question. Testing for expression of an integrated viral genome in diverse differentiated tissues may provide a useful model system to study the regulation of differentiation.

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